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Granulocyte-colony Stimulating Factor and Retinoic Acid Cooperatively Induce Granulocytic Differentiation of Acute Promyelocytic Leukemia Cells *in vitro*

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The interaction of granulocyte-colony stimulating factor (G-CSF) and retinoic acid (RA) in proliferation and differentiation of acute promyelocytic leukemia (APL) cells was examined. G-CSF stimulated proliferation of APL cells at concentrations of 0.1 to 50 ng/ml in a dose dependent manner. More than $10^{-8}M$ RA induced granulocytic differentiation of APL cells. Although G-CSF induced lysozyme activities in APL cells, it alone did not induce terminal differentiation of APL cells. G-CSF significantly enhanced the RA-induced granulocytic differentiation of APL cells *in vitro*. Enhancement by G-CSF was not due to the prolongation of survival of RA-induced differentiated cells, but the differentiation-inducing effects of G-CSF might be evident only in the presence of RA. Since G-CSF has a potential to induce the granulocytic differentiation of myeloid leukemia cells, G-CSF in combination with RA may be applicable in differentiation induction therapy for some types of myeloid leukemia.

Key words: Granulocyte-colony stimulating factor — Retinoic acid — Myeloid leukemia cell differentiation

G-CSF is a hemopoietic growth factor that controls proliferation and differentiation of normal granulocyte progenitor cells.[1)] It is also known to act on proliferation and differentiation of myeloid leukemia cells. It was reported that murine G-CSF induced the terminal differentiation of the murine myelomonocytic leukemia cell line, WEHI-3B.[2)] G-CSF initially stimulated proliferation of the human promyelocytic leukemia cell line, HL-60, but subsequently induced differentiation with suppression of clonogenicity.[3)] Some investigators reported that human myeloid leukemia cells from patients were induced by G-CSF to undergo terminal differentiation to macrophages and granulocytes,[4)] but others reported that freshly obtained myeloid leukemia cells were stimulated by G-CSF to proliferate rather than differentiate.[5)] So far, there has been disagreement among various authors concerning the differentiation-inducing effects of G-CSF on human myeloid leukemia cells.

In the present study, to elucidate the role of human G-CSF in the granulocytic differentiation of human myeloid leukemia cells, we investigated the interaction of RA, known to be a potent inducer of granulocytic differentiation of human myeloid leukemia cells,[6)] and G-CSF in the proliferation and differentiation of APL (M3) cells.

Abbreviations: G-CSF, granulocyte-colony stimulating factor; RA, all-*trans*-β-retinoic acid; APL, acute promyelocytic leukemia; NBT, nitroblue tetrazolium; ^{3}H-TdR, tritiated thymidine; PBS, Ca^{2+}/Mg^{2+}-free phosphate-buffered saline.

MATERIALS AND METHODS

Patient details Three patients were diagnosed with APL (M3) according to the French-American-British classification.

Case 1. T.N., a 49-year-old woman, was admitted because of genital bleeding in July 1988. Hematological data were as follows: RBC$=4.32\times10^{12}$/liter; WBC$=0.9\times10^{9}$/liter; with 6% promyelocytes; platelets$=10.4\times10^{9}$/liter. Bone marrow was massively infiltrated by promyelocytes, 81.2%. The karyotype was 46,XX,-15, -17,+der(15)t(15;17)(q22;q21),+i(17q$-$) in 100% of bone marrow metaphases.

Case 2. N.A., a 71-year-pld woman, had been diagnosed as having APL in August 1988. Complete remission was achieved after chemotherapy. In January 1989 she suffered from high fever. The total WBC count was 59.6×10^{9}/liter with 92% promyelocytes and bone marrow differential was 94.4% promyelocytes at relapse. Cytogenetic studies showed 46,XX,t(15;17)(q22;q21) at diagnosis and 46,XX,-6,+der(6)t(6;?)(q23;?),t(15;17)(q22;q21) at relapse.

Case 3. K.K., a 25-year-old man, presented with loss of consciousness due to multiple cerebral hemorrhage in November 1988. He was diagnosed as having variant APL following an electron microscopic study. The total WBC count was 217.8×10^{9}/liter with 96% promyelocytes. Bone marrow was hypercellular with 94.8%

promyelocytes. The karyotype was 46,XY,t(15;17)(q22;q21),+mar in 100% of metaphases.

Cell preparations Promyelocytic leukemia cells were harvested from the bone marrow (case 1) or peripheral blood (case 2 at relapse, case 3) of these 3 APL patients. Leukemia cells were isolated by a standard centrifugation step with Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). The number of cells was counted with a cell counter (Microcell Counter CC-130, Sysmex, Tokyo) and viability was assessed by trypan blue dye exclusion.

DNA synthesis APL cells (3×10^5 cells/ml) were cultured for 72 h in 1 ml of RPMI 1640 culture medium (Gibco, Grand Island, NY) with 15% FCS per well in 24-well plates (Nunc) in the presence of various concentrations of RA and G-CSF. The cells were labeled with 10 μCi per well of ^{3}H-TdR (6.7 Ci/mmol, New England Nuclear) for the last 16 h of incubation, then resuspended and washed with PBS. The precipitates were collected on a membrane filter, and washed with 5% trichloroacetic acid, then the radioactivity was counted in Aquasol-2 (New England Nuclear) in a liquid scintillation counter (Aloka, LSC-903).

Induction of differentiation of APL cells For induction of differentiation of promyelocytic leukemia cells, cell suspensions containing more than 90% leukemia cells were plated in 60×10 mm plastic dishes (Lux 5520) at 5×10^5 cells/ml and incubated for 7 days in RPMI 1640 culture medium supplemented with 15% heat-inactivated fetal calf serum (FCS) (Gibco) at 37°C in a humidified atmosphere of 5% CO_2 in air. RA (Sigma Chemical Co., St. Louis, MO) was suspended in absolute enthanol at concentrations of 10^{-2} *M* to 10^{-7} *M* and stored at −20°C in foil-wrapped tubes. It was added to cultures under subdued light. Recombinant G-CSF (Chugai Pharmaceutical Company, Tokyo) was stored at −80°C in PBS containing 0.1% human serum albumin. G-CSF had a specific activity of about 5×10^7 U/mg protein.

Differentiation associated properties For morphological examination, cytospine slides were stained with May-Grünwald Giemsa and observed through a light microscope. Differential counts were performed on 200 cells.

NBT (Sigma) reduction was assayed using the technique described by Honma *et al.*[7] The percentage of cells reducing NBT was determined by counting at least 200 cells.

Lysozyme activity was determined using the lysoplate method described by Kasukabe *et al.*[8]

The percentage of cells expressing differentiation surface antigen was determined by indirect immunofluorescence (flow cytometry) using the monoclonal antibody, OKM1, which is specific for mature myelomonocytic cells.[9] Cells (4×10^5) were treated with OKM1 (Ortho Pharmaceutical Co., Raritan, NJ), incubated for 30 min at 4°C, and washed twice with PBS containing 0.2% bovine serum albumin and 0.1% NaN_3. A control sample was treated with mouse IgG1 (Coulter Immunology, Hialeah, FL). The cells were then incubated with fluorescein isothiocyanate-conjugated goat $F(ab')_2$ anti-mouse $F(ab')_2$ antiserum (Ortho) for 30 min at 4°C. The percentage of fluorescent cells was determined in a flow cytometer (Becton Dickinson, FACS Division, Sunnyvale, CA). The threshold fluorescence intensity used was that at which 98% of the control cell population was negative.

RESULTS

Effects of G-CSF and RA on uptake of ^{3}H-TdR in APL cells G-CSF augmented ^{3}H-TdR uptake in APL cells from all 3 cases at concentrations of 0.1 to 50 ng/ml in a dose-dependent manner (Fig. 1). The addition of 10^{-7} *M* RA produced inhibition in 2 cases (Fig. 1, cases 1 and 2) and enhancement in one case (Fig. 1, case 3) on ^{3}H-TdR uptake of APL cells stimulated by G-CSF.

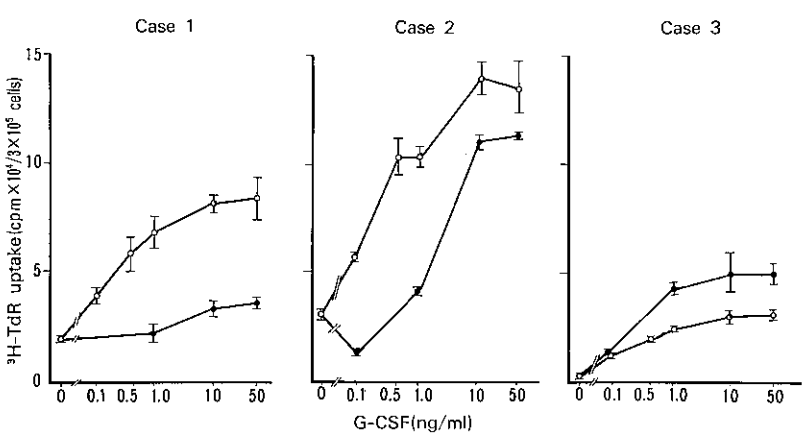

Fig. 1. G-CSF-induced ^{3}H-TdR uptake in APL cells from the 3 cases in the presence (●) or absence (○) of 10^{-7} *M* RA. Data are expressed as mean cpm±SD of triplicate cultures.

No significant uptake of ^{3}H-TdR was observed in APL cells from cases 1 and 3 either in the presence or absence of RA (Fig. 2). In case 2 (Fig. 2), uptake of ^{3}H-TdR was inhibited by RA at concentrations higher than 10^{-8} *M*. G-CSF-stimulated ^{3}H-TdR uptake was markedly inhibited by more than 10^{-8} *M* RA in APL cells from case 1 and case 2 (Fig. 2). However, ^{3}H-TdR uptake of APL cells from case 3 was enhanced by RA at 10^{-9} *M* to 10^{-7} *M* in the presence of 1.0 ng/ml G-CSF (Fig. 2).

Effects of RA and G-CSF on the induction of differentiation of APL cells G-CSF (0.1 to 50 ng/ml) alone did not induce NBT reduction of APL cells from the 3 cases. RA induced NBT reduction of APL cells from all the cases at concentrations higher than 10^{-8} *M*. Enhancement of RA-induced NBT reduction of APL cells by G-CSF depended on the concentrations of RA. Although 10^{-9} *M* RA alone did not induce differentiation of APL cells, in combination with G-CSF, increase of NBT-positive cells was obviously induced by 10^{-9} *M* RA in APL cells from 3 cases (Fig. 3). Table I summarizes the induction of differentiation of APL cells (cases 1 and 3) by RA, G-CSF and their combinations. RA at 10^{-7} *M* induced most APL cells from cases 1 and 3 into intermediate granulocytes (myelocytes, metamyelocytes) accompanied with increases of NBT reduction and lysozyme activities. G-CSF at 10 ng/ml induced lysozyme activities in APL cells. However, G-CSF alone could not induce obvious granulocytic differentiation of APL cells as judged by morphological examinations. RA and G-CSF showed marked cooperative differentiation-inducing effects on APL cells. The terminally differentiated granulocytes (stab, segmented cells) could be induced only by RA plus G-CSF (Fig. 4). In case 2 (data not shown), similar effects of RA and G-CSF were observed.

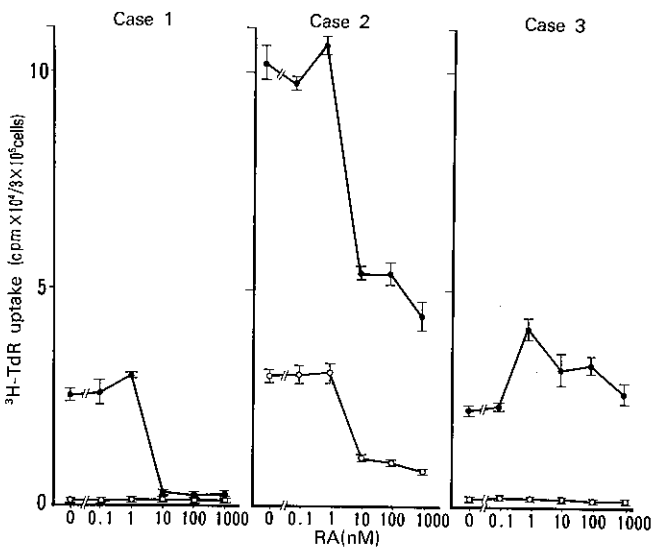

Fig. 2. Effects of RA on ^{3}H-TdR uptake in APL cells from the 3 cases in the presence (●) or absence (○) of 1.0 ng/ml of G-CSF. Data are expressed as mean cpm±SD of triplicate cultures.

Fig. 5 shows the expression of differentiation surface antigen of APL cells from 3 cases induced by RA and G-CSF. There was no significant expression of OKM1 on the surface of noninduced APL cells from the 3 cases (data not shown). G-CSF at 0.1 to 10 ng/ml did not induce OKM1-expression on APL cells from the 3 cases. In case 1, an increase of OKM1-positive cells was obvious with 10^{-7} *M* RA, and NBT reduction of the APL cells was also induced. In cases 2 and 3, 10^{-7} *M* RA did not induce a significant increase of OKM1-expression while RA at the same concentration remarkably induced NBT reduction in these APL cells. In cases 2 and 3, G-CSF dose-dependently induced OKM1-expression in the presence of RA.

Effects of G-CSF and RA on the survival of differentiation-induced APL cells The number of RA-induced NBT-positive APL cells decreased within 3 days after incubation in a culture medium without inducers. In the treatment with G-CSF, the number of NBT-positive cells increased slightly on day 3 and then decreased rapidly. In the treatment with G-CSF plus RA, the number of

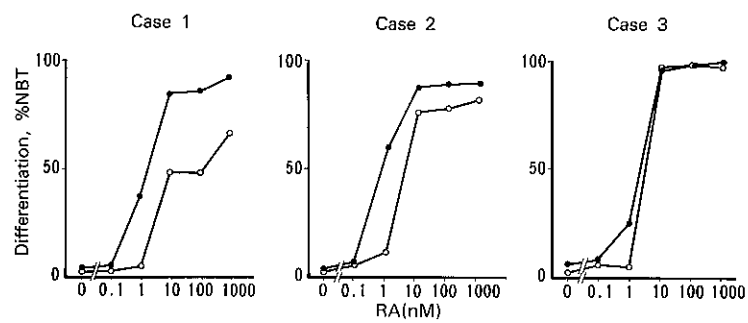

Fig. 3. Differentiation of APL cells from 3 cases treated with various concentrations of RA in the presence (●) or absence (○) of 1.0 ng/ml of G-CSF. The percentage of cells reducing NBT was determined after 7 days of incubation.

Table I. Induction of Differentiation of APL Cells by RA and G-CSF

	Treatment	No. of cell ($\times 10^5$/ml)	Morphological changes (%)						NBT-positive cells (%)	Lysozyme (U/10^7 cells)
			Pro	Myl	Met	St	Seg	Mon		
Case 1	Before	5.0	100						0	10.0
	Medium	5.7	99	1					0	46.7
	RA 10^{-7} *M*	4.8	1	95	3.5		0.5		45	172.1
	G-CSF 10 ng/ml	8.6	96	4					13	117.3
	RA $10^{-7}M$ + G-CSF 10 ng/ml	4.8	1.5	9	68	10	11.5		93	264.1
Case 3	Before	5.0	98.5	1.5					0	26.0
	Medium	5.4	97.5	2.5					3	82.5
	RA $10^{-7}M$	4.5	5	69	26				90	139.0
	G-CSF 10 ng/ml	6.7	100						10	128.6
	RA 10^{-7} *M* + G-CSF 10 ng/ml	7.6	2.5	12.5	65.5	11.5	7	1	96	261.3

Pro, promyelocytes; Myl, myelocytes; Met, metamyelocytes; St, stab; Seg, segmentation; Mon, monocytes.

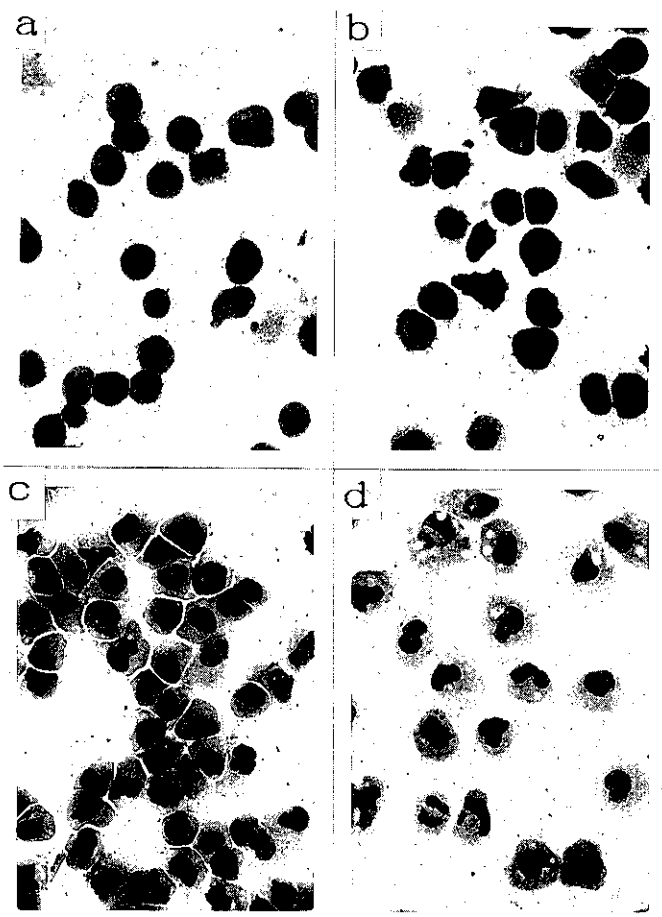

Fig. 4. Morphology of APL cells from case 1. The APL cells were cultured for 7 days in: (a) culture medium, or in culture medium with (b) 10 ng/ml of G-CSF; (c) 10^{-7} *M* RA; (d) 10 ng/ml of G-CSF plus 10^{-7} *M* RA.

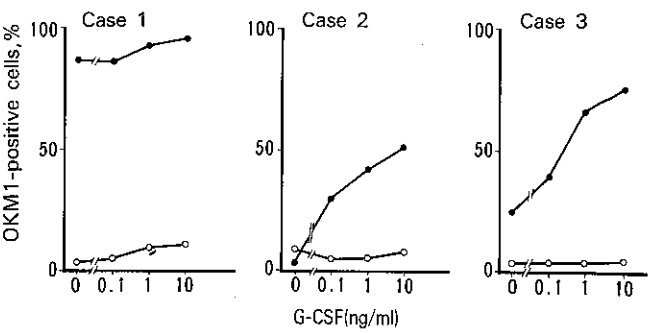

Fig. 5. Effects of G-CSF and RA on the surface antigen expression of differentiation of APL cells. APL cells from the 3 cases were treated with various concentrations of G-CSF in the presence (●) or absence (○) of 10^{-7} *M* RA and incubated for 7 days. The percentage of OKM1-positive cells was determined as described in "Materials and Methods."

NBT-positive cells was maintained more consistently (Fig. 6). The effects of RA plus G-CSF on the RA-induced differentiated cells were confirmed by morphological examination (data not shown). Most APL cells from case 2 were induced to differentiate into intermediate granulocytes in 7 days by treatment with 10^{-7} *M* RA. After incubation for 7 additional days without any inducer, the number of viable cells decreased and further morphological maturation was not induced. G-CSF alone also did not affect the differentiation or survival of RA-induced intermediate granulocytes. Terminally differentiated granulocytes could be induced only by simultaneous addition of RA plus G-CSF.

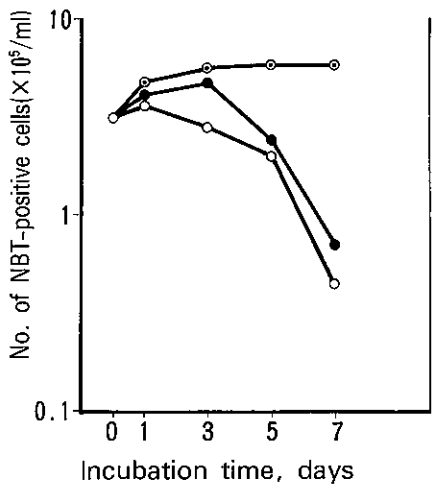

Fig. 6. Effects of G-CSF and RA on the survival of differentiation-induced APL cells. APL cells from case 2 (5×10^5 cells/ml) were cultured for 7 days in the presence of 10^{-7} *M* RA, washed, and resuspended in the culture medium. The cells (5×10^5 cells/ml) were then incubated without inducers (○) or with 10 ng/ml of G-CSF in the presence (◉) or absence (●) of 10^{-7} *M* RA. At the indicated days, the numbers of cells reducing NBT were determined.

DISCUSSION

The present results show that G-CSF not only stimulated proliferation but also markedly enhanced RA-induced granulocytic differentiation of freshly obtained APL cells *in vitro*. Although G-CSF induced some properties related to differentiation of APL cells, it alone could not induce terminal differentiation of APL cells even at concentrations as high as 50 ng/ml. However, its differentiation-enhancing effects in combination with RA were sufficiently demonstrated at concentrations lower than 10 ng/ml, at which colony formations from normal granulocyte progenitor cells were also supported.[1] Although the differentiation enhancement by G-CSF depended on the concentration of RA, this effect was not merely a prolongation of survival of already differentiated APL cells induced by RA. Further, in two cases, G-CSF as well as RA was necessary for the induction of differentiation surface marker, OKM1, on APL cells. It is supposed that G-CSF has a potential to induce differentiation of APL cells, but the differentiation-inducing effects of G-CSF might be evident only in the presence of RA.

The interactions of G-CSF and RA in the proliferation of APL cells were complicated. It has been reported that RA inhibited clonal growth of freshly isolated myeloid leukemia cells supported by phytohemagglutinin-stimulated leukocyte-conditioned medium (PHA-LCM) and these inhibitory effects were not related to differentiation-inducing effects of RA.[10] The inhibitory effects of RA on the proliferation of APL cells may be closely related to its differentiation-inducing effects, because the inhibition of ^{3}H-TdR uptake was found only at concentrations greater than 10^{-8} *M*, at which APL cells were also induced to differentiate. In one case (case 3), G-CSF in the presence of RA simultaneously stimulated the proliferation and differentiation of APL cells *in vitro*. The distinctive effects of G-CSF on APL cells from case 3 may be understood from the essential actions of CSFs to support the growth and differentiation of hemopoietic progenitor cells.[11] RA may modify the response of APL cells to G-CSF in proliferation and differentiation by binding to a specific retinoic acid receptor.

It has been pointed out previously that G-CSF showed clear proliferative effects on human myeloid leukemia cells, especially on APL cells *in vitro*, and injected G-CSF might cause the proliferation of leukemia cells *in vivo*.[12, 13] Our findings show that the proliferative stimuli of G-CSF on human myeloid leukemia cells were sufficiently inhibited by physiological concentrations of RA[14] in some cases. Further, despite the proliferative ability of G-CSF, G-CSF in the presence of RA revealed obvious differentiation-inducing effects on human myeloid leukemia cells.

Since G-CSF is important for the granulocytic differentiation of myeloid leukemia cells, in combination with RA it may be applicable in differentiation induction therapy for some types of myeloid leukemia.

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